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Comparison of Plasma Levels of Cytokines and In Vitro Generation of Reactive Oxygen Species After Nicotine Infusion in Nicotine Users with Normal and Impaired Renal Function

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ABSTRACT

Several *in vitro* and animal studies suggest effects of nicotine on the immune system, but little evidence exists regarding the *in vivo* immunomodulation of nicotine in humans. The increased use of nicotine replacement therapy to aid smoking cessation claims further understanding of how nicotine affects blood leukocytes. This is of particular importance when nicotine therapy is used in diseases associated with alterations of the immune system, such as chronic renal failure. The present study evaluates the acute effects of nicotine infusion (NI) on some immunoregulatory functions in seven healthy subjects and seven patients with renal failure. All subjects were nicotine users and had refrained from using nicotine for 36 h before NI. Blood was collected before, immediately after, and 2 h after NI. Plasma concentrations of intercellular adhesion molecule-1 (ICAM-1) and the cytokines interleukin-2 (IL-2), IL-4, IL-10, interferon-γ and RANTES were measured using specific immunoassays. The generation of reactive oxygen species (ROS) induced by formyl-methionyl-leucyl-phenylalanine (fMLP), Ristocetin, adenosine 5′-diphosphate, or collagen was registered in whole blood as luminol-dependent chemiluminescence. Except for fMLP, these compounds induce

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leukocyte ROS generation by platelet mediated mechanisms. NI did not significantly affect the levels of the cytokines and ICAM-1 in any group. The peak and the persistent ROS production, induced by collagen and Ristocetin, was lower at some time points in patients with renal failure as compared to healthy subjects. Also in patients with renal failure, both peak height and persistent ROS generation induced by Ristocetin were reduced immediately after NI. Thus, nicotine inhibits some of the platelet-mediated activation of leukocyte ROS generation, and may be associated with platelet defects in renal failure.

Key Words: Nicotine; Immunomodulation; Platelet–leukocyte interactions; Reactive oxygen species; Cytokines; Renal failure.

Abbreviations: ADP, adenosine 5′-diphosphate; fMLP, formyl-methionyl-leucyl-phenylalanine; IL, interleukin; IFN-γ, interferon-γ; NI, nicotine infusion; PMCs, human peripheral blood mononuclear cells; ROS, reactive oxygen species.

INTRODUCTION

Cigarette smoking is associated with an increased prevalence of cardiovascular disease, cancer and several other disorders. Although smoking also is known to have detrimental effects on the immune system, the nature of the immunosuppression is poorly understood.[1,2] Reported data of the immunoregulatory effects of the major active component of tobacco products, nicotine, are often conflicting.

Cytokines have a central role in influencing the type of immune response needed for optimal protection against infectious agents but inappropriate production of cytokines is involved in the pathology of various inflammatory diseases.[3] *In vitro*, nicotine has been reported to increase human peripheral blood mononuclear cells (PMCs) production of mRNA coding for IL-2 and IL-4 as well as other cytokines.[4] Furthermore, PMCs from smokers produce higher levels of IL-4 *in vitro* as compared to PMCs from non-smokers.[5] However, nicotine suppressed the production of IL-2 and interferon-γ (IFN-γ) in other studies,[6,7] and one report concluded that nicotine does not modify IL-4 and IFN-γ release.[8] In healthy non-smokers, nicotine patch treatment inhibited the PMC production of IL-10, but not IL-2.[9] In another study, treatment of healthy non-smoking volunteers with nicotine patches inhibited both IL-2 and IL-10 production.[10]

IL-2, IL-4, IL-10 and IFN-γ are produced by T-cells and other leukocytes, and regulate the activation of leukocytes.[3,11] Since these cytokines appear to be involved in the immunoregulatory effects of nicotine *in vitro*, these messengers were analyzed in the present study together with ICAM-1 and RANTES. Endothelium, epithelium, monocytes, T- and B-cells and dendritic cells express the adhesion molecule ICAM-1, which mediates the adhesion to leukocytes.[11] ICAM-1 is considerably elevated in cigarette smokers and dramatically declines upon quitting smoking.[12] RANTES, produced by platelets and T-cells, is a chemokine that attracts T-cells, eosinophils and monocytes.[11] *In vitro* studies have shown nicotine to be a chemoattractant to neutrophils, and in lower concentrations to enhance the response to chemotactic peptides.[13]

The use of nicotine replacement therapy to aid smoking cessation is increasing,[14] and nicotine is also considered to have beneficial effects in ulcerative colitis, Parkinson's and Alzheimer's disease.[15] Consequently, it is of great importance to evaluate the effects of nicotine on blood leukocytes, particularly when nicotine therapy is used in diseases associated with alterations of the immune system and infection susceptibility. In chronic renal failure or uremia, dysfunctions are reported in the T- and B-cells in the specific immune defence,[16] as well as in the polymorphonuclear granulocytes, the main cells of the unspecific defence system during infections.[17] Granulocytes from uremic patients show elevated basal levels of cytosolic calcium, reduced ROS generation during respiratory burst, and impaired phagocytosis. Upon interaction with soluble or particulate stimuli, the granulocytes increase their oxygen consumption to generate reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide. This respiratory burst can be measured using a luminol-dependent chemiluminescence method. In addition to the role of ROS as a killing mechanism for infectious agents upon phagocytosis, ROS generation may have a role in the mechanisms of atherosclerosis and thrombosis.[18]

We have recently reported that renal failure is associated with decreased nicotine elimination,[19] and that nicotine infusion (NI) increases platelet P-selectin expression in nicotine users with normal as well as with impaired renal function.[20] P-selectin, which is expressed on platelets and endothelial cells upon activation, is an adhesion receptor for several types of leukocytes. Consequently, platelet P-selectin appears to be a link between the hemostatic and the inflammation process.[21] Indeed, we have previously reported that platelets recruit and increase the chemotactic migration of neutrophils through P-selectin dependent mechanisms.[22] The objective of the present study was to investigate if the observed platelet activation upon NI concurs with altered cytokine levels in plasma or influences some leukocyte functions. Consequently, ROS generation was induced by compounds that primarily activate platelets, which in turn activate leukocytes, and one compound that acts directly on leukocytes.

METHODS

Study Population, Nicotine Infusion, and Collection of Blood Samples

Seven healthy subjects (41–59 years, mean 48) and seven patients with renal failure (42–57 years, mean 51) participated in the study after given informed consent, see previous report[20] for complete demographics. Both controls and patients were excluded if they had cardiovascular abnormalities, established liver disease, or other intercurrent illness (besides renal impairment). The study conforms to the principles outlined in the Declaration of Helsinki, Finland 1964 and later revisions, and was approved by the local ethical committee (Linköping, Sweden). The mean and range of glomerular filtration rate (GFR) was 109 (84–143) mL/min in the control group and 16 (6–36) in the group of patients with renal failure. The GFR was determined by ^{51}CrEDTA-clearance, except in 3 patients treated with continuous ambulatory peritoneal dialysis (CAPD). In these patients total clearance (dialysate + renal clearance) was assessed by the mean of urea and creatinine 24-hour clearances in urine and dialysate. Leukocyte and platelet count and hemoglobin levels were measured in each subject not more than one month prior to

the start of the study, and there were no significant differences between the groups. Both controls and patients were cigarette smokers (10–20 cigarettes daily) or used wet snuff (20–50 g daily). Reported consumption was determined from patient recall. All subjects had refrained for 36 hr from using nicotine before administration of an iv infusion of nicotine (NI) over 10 min (0.028 mg/kg body wt). The volume infused was 0.1 mL/kg body wt. Blood collection was performed immediately before and after NI, and 2 hr after the start of NI. Because a circadian variation of platelet function has been reported,[23,24] NI was started at 8.00 a.m. in all subjects.

Assays for IL-2, IL-4, IL-10, IFN-γ, ICAM-1, and RANTES in Plasma

Blood was collected in 5-mL Diatube-HR tubes (CTAD, sodium Citrate/ Theophylline/ Adenosine/ Dipyridamole, from Becton Dickinson, Oxford, UK) and immediately placed in ice water. Within 30 min, the tubes were centrifuged for 30 min at 2,500 *g* in 4°C, plasma was withdrawn and stored at − 70°C until assayed. Levels of IL-2, IL-4, IL-10, IFN-γ and sICAM-1 were measured using immunoassays from Amersham Life Science, Buckinghamshire, UK. RANTES was determined by an immunoassay from R&D Systems, Abingdon, UK. All assays were performed according to standard procedures.

Measurement of Reactive Oxygen Species in Whole Blood

The generation of reactive oxygen species (ROS) in whole blood was detected by luminol-amplified chemiluminescence[25] in a 2-channel Lumi-aggregometer (Chrono-Log Corporation, Havertown, PA, USA). Blood samples were anticoagulated with heparin and diluted with an aliquot of normal saline. Samples were incubated at 37°C for 5 min in the presence of 50 μM luminol (Sigma Chemical Co., St. Louis, MO, USA) prior to stimulation with 1 μM formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma Chemical Co.), 5 μM adenosine 5′-diphosphate (ADP, Sigma Chemical Co.), 0.62 mg/mL Ristocetin (Diagnostica Stago, Asnières-sur-Seine, France), or 1.25 μg/mL collagen (Chrono-Log Corp., Havertown, PA, USA). Data are based on changes in relative light units (RLU) of chemiluminescence recorded over a period of 15 min. The peak height, the peak time and the persistent light emission after 10 min were evaluated.

Statistics

All assays were conducted with samples in duplicate. The mean of the duplicates was used for calculations and results are expressed as mean ± SEM. Statistical analysis was performed in GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA). For within-group comparisons, the values in each group were analyzed with Friedman two-way analysis of variance by ranks, followed by Dunn's post test. For between-group comparisons, the values in each group were analyzed with two-tailed Mann-Whitney test. A p value of 0.05 or less was judged as statistically significant.

RESULTS

Cytokines and ICAM-1 in Plasma

NI did not significantly alter the plasma levels of IL-2, IL-4, IL-10, IFN-γ, RANTES and soluble ICAM-1, and there were no significant differences between the groups (Table 1). However, there was a tendency of lower IL-2 and IL-10 levels in healthy individuals directly after NI compared to baseline levels ($p = 0.06$ and 0.08 respectively), but after 2 h this tendency was lost. Only one sample, from a healthy subject at baseline, contained a detectable concentration of IL-4 (data not shown).

Generation of Reactive Oxygen Species in Whole Blood

Addition of the platelet agonists ADP, collagen or Ristocetin to whole blood triggered significant aggregation and ROS generation, whereas addition of the chemotactic peptide fMLP only caused ROS generation. The aggregation data has previously been reported.[20] The magnitude (peak height) and the duration (10 min chemiluminescence) of the response were similar for the different stimuli. Maximal chemiluminescence (peak time) was reached after various times. The peak time was significantly shortest with fMLP (range of mean was 55–60 sec), intermediate with collagen (145–198 sec) and ADP (192–240 sec), and longest with Ristocetin (326–444 sec). However, the peak times of the different activators were not significantly different between the groups and NI did not alter them (Fig. 1). The collagen-induced generation of ROS was significantly lower in patients

Table 1. Plasma levels of IL-2, IL-10, IFN-γ, sICAM-1, and RANTES in seven healthy subjects and seven patients with renal failure. The levels were measured directly before nicotine infusion (Baseline), immediately after nicotine infusion, and 2 h after nicotine infusion. Data are expressed as means ± SEM.

	Baseline	Immediately after nicotine infusion	2 h after nicotine infusion
Healthy subjects			
IL-2 (pg/mL)	13.89 ± 7.03	7.69 ± 4.25	12.56 ± 4.78
IL-10 (pg/mL)	14.35 ± 3.52	7.02 ± 1.78	13.16 ± 3.48
IFN-γ (pg/mL)	0.46 ± 0.46	0.19 ± 0.19	0.94 ± 0.45
sICAM-1 (ng/mL)	52.58 ± 4.62	53.36 ± 4.77	49.10 ± 7.59
RANTES (ng/mL)	4.96 ± 2.79	6.02 ± 2.96	6.38 ± 3.13
Patients with renal failure			
IL-2 (pg/mL)	9.71 ± 3.59	14.56 ± 5.97	8.40 ± 4.92
IL-10 (pg/mL)	10.24 ± 3.08	9.69 ± 4.44	8.22 ± 2.42
IFN-γ (pg/mL)	0.62 ± 0.29	0.48 ± 0.46	0.38 ± 0.37
sICAM-1 (ng/mL)	48.15 ± 2.63	50.05 ± 2.81	50.63 ± 3.44
RANTES (ng/mL)	3.37 ± 0.64	2.73 ± 0.34	2.65 ± 0.52

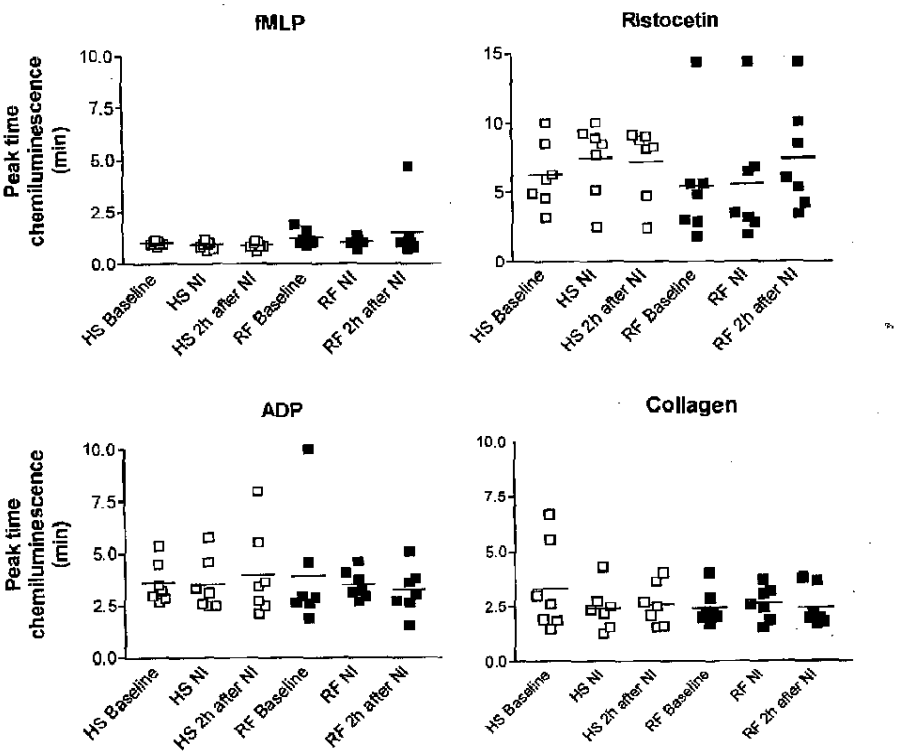

Figure 1. Effects of nicotine infusion (NI) on the rate of generation of reactive oxygen species (ROS) in blood from seven healthy subjects (HS, open squares) and seven patients with renal failure (RF, closed squares). Peak time (minutes) chemiluminescence (relative light units, RLU) denoting generation of ROS was measured directly before NI (Baseline), immediately after NI (NI), and 2 h after NI. ROS generation was induced by fMLP, Ristocetin, ADP, and collagen. Horizontal lines represent the mean value.

with renal failure, as compared to healthy individuals, before and immediately after NI measured as peak height, and immediately after NI measured as the ROS generation 10 min after activation (Figs. 2 and 3).

The peak of ROS generation induced by Ristocetin was significantly inhibited directly after NI in blood from patients with renal failure (Fig. 2). At peak time, approximately 5–7 min after activation, the ROS production was also significantly lower in the patient group as compared to healthy individuals. This alteration reversed and was not longer different to baseline values after 2 h. Thus, since the Ristocetin-induced peak of ROS generation was late, the chemiluminescence at 10 min was also significantly lower compared to baseline (Fig. 3). NI did not significantly alter ROS generation induced by fMLP, ADP or collagen.

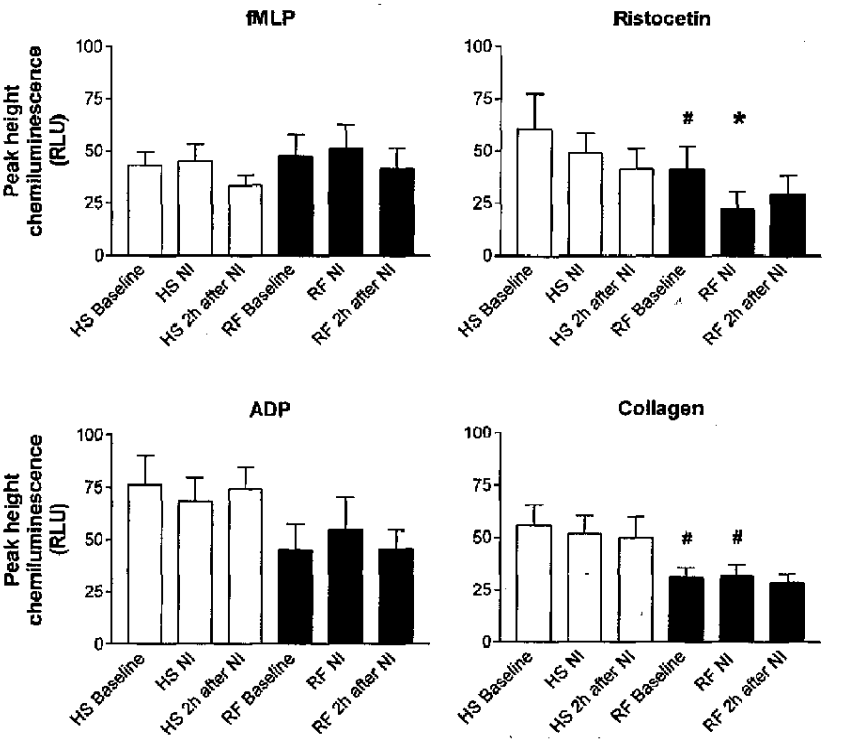

Figure 2. Effects of nicotine infusion (NI) on maximal generation of reactive oxygen species (ROS) in blood from seven healthy subjects (HS, white bars) and seven patients with renal failure (RF, black bars). Peak height chemiluminescence (relative light units, RLU) denoting generation of ROS was measured directly before NI (Baseline), immediately after NI (NI), and 2 h after NI. ROS generation was induced by fMLP, Ristocetin, ADP, and collagen. Data are expressed as means± SEM. $^{*}p < 0.05$ vs. baseline within group, and $^{\#}p < 0.05$ vs. HS (between groups).

DISCUSSION

Several studies have demonstrated that nicotine has immunomodulatory effects. The current investigation focus, for the first time, on acute effects of a nicotine infusion (NI) in nicotine users with normal and impaired renal function. The parameters studied were the plasma levels of IL-2, IL-4, IL-10, IFN-γ, RANTES and ICAM-1. In addition, induced ROS generation in whole blood was analyzed. NI did not significantly alter the levels of the cytokines or the adhesion molecule ICAM-1, and there were no differences between the two groups. The basal production of IFN-γ was near zero pg/mL in most of the subjects, and IL-4 was only detectable in one sample. Consequently, a suppressive action of nicotine on these cytokines could not be detected. In accordance, the manufacturer of the immunoassays, used in the present study, indicates that the normal average levels of

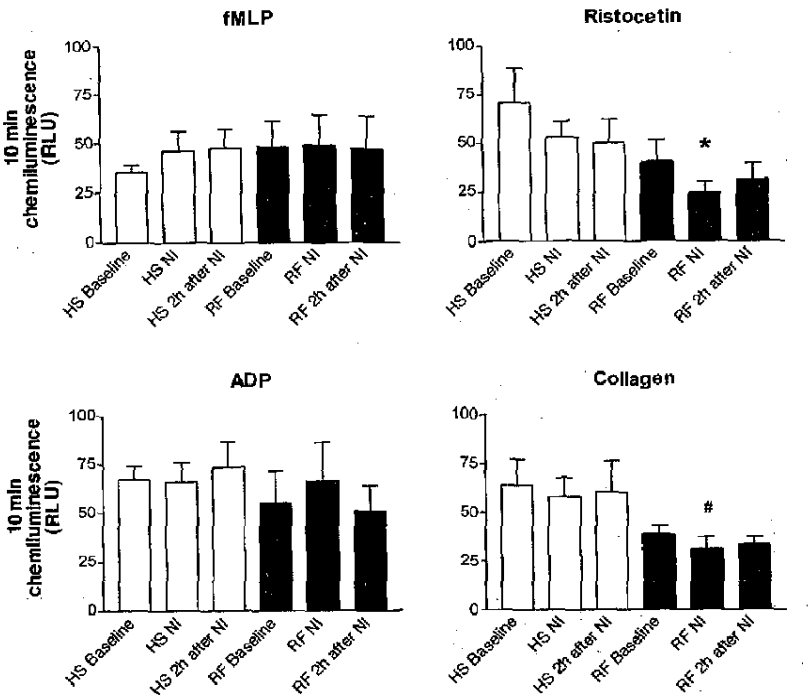

Figure 3. Effects of nicotine infusion (NI) on persistent generation of reactive oxygen species (ROS) in blood from seven healthy subjects (HS, white bars) and seven patients with renal failure (RF, black bars). Persistent chemiluminescence (relative light units, RLU) denoting generation of ROS 10 min after induction was measured directly before NI (Baseline), immediately after NI (NI), and 2 h after NI. ROS generation was induced by fMLP, Ristocetin, ADP, and collagen. Data are expressed as means ± SEM. $^{*}p < 0.05$ vs. baseline within group, and $^{\#}p < 0.05$ vs. HS (between groups).

IFN-γ and IL-4 in plasma are 0.3 and 0 pg/mL, respectively. The plasma levels of ICAM-1 in the present study were approximately 7-fold lower than previously reported in continuing smokers.[12] On the contrary, the levels of RANTES in the present study were approximately 5-fold higher than the normal average levels for heparin plasma, stated by the manufacturer of the immunoassay. Cytokines levels in plasma are significantly affected by the anticoagulant used during blood collection.[26] The molecules can be degraded as well as released during the processing of blood. There are, by our knowledge, no previous reports where the mixture of sodium citrate/theophylline/adenosine/dipyridamole (CTAD) is used. CTAD appears to be superior to other more commonly used anticoagulants regarding platelet and leukocyte activation.[27,28] The plasma levels of RANTES in the present study are comparable with the levels in a previous study where CTAD was used upon blood collection.[29]

Both in whole blood and in PMC suspensions, significant inter-individual variations have been found in the production of cytokines like IL-2, IL-10 and IFN-γ, and the levels appear to be individually characteristic.[30] Nicotine patch treatment has earlier been reported to inhibit IL-10 production.[9] In the present study, NI decreased the IL-10 levels in all but three subjects, one healthy and two renal impaired subjects. Only these three subjects had lower IL-10 levels than 1 pg/mL at baseline, and NI increased these IL-10 levels. There was also a tendency of lower IL-2 levels in healthy individuals immediately after NI. According to the manufacturer of the immunoassays, the normal average levels of IL-2 and IL-10 in plasma are 3.6 and 4.5 pg/mL, respectively.

In the current study there were no indications that nicotine, administrated *in vivo*, acutely affected the basal production of any of the soluble mediators measured in whole blood. These findings cannot exclude any action of nicotine on induced cytokine production by PMCs. Further, our results do not reveal anything about the long-term effects of nicotine on immunomodulation in humans. The acute immunomodulation of nicotine has been reported to be mediated through different pathways than those involved during chronic nicotine treatment.[31] Several findings also point towards alternative compounds to nicotine as major actors in the noxious effects in cigarette smoking.[7,32,33]

The granulocytes play a major role in the first defence against infections, partly due to their ability to generate ROS. In the present study, ROS generation was induced by four different stimuli acting through different mechanisms and with various lag phases. fMLP is a widely used inducer of the respiratory burst in neutrophil granulocytes, and acts through binding to specific cell surface, G-protein linked, receptors.[34,35] Previous *in vitro* studies on the direct effects of nicotine on ROS production in leukocytes, i.e. induced by fMLP, report no effect,[13] potentiation,[36] as well as inhibition.[37] It has also been reported that cigarette smoking has no effect on the generation of ROS, measured by chemiluminescence in whole blood.[35]

Collagen as well as ADP and Ristocetin probably elicit ROS generation in whole blood via platelet activation, although collagen has been shown to bind and activate neutrophils directly through the integrin LFA1.[38] Previous experiments have shown that collagen dose-dependently induces ROS generation in whole blood but not in pure neutrophil suspensions.[39] Also platelets have the ability to produce ROS, but much less than leukocytes[40] and not in the same burst-like manner.[18] Thus, since ADP, collagen and Ristocetin are known as strong platelet activators, with few powerful direct effects on leukocytes, these agents probably activate leukocyte ROS generation via platelet activation. The mechanism by which activated platelets induce ROS production by granulocytes as well as monocytes is very likely mediated by P-selectin.[41] This adhesive ligand for several types of leukocytes is expressed on platelets and endothelial cells upon activation, and forms a link between hemostatic and inflammatory processes.[21] Platelets also recruit and increase the chemotactic migration of neutrophils through P-selectin dependent mechanisms.[22]

In the present study, the peak height of collagen-induced ROS production was significantly lower in patients with renal failure compared to healthy subjects before and immediately after NI (2 h after NI the p value was 0.064). The p values for 10 min chemiluminescence were 0.082, <0.05, and 0.064 for baseline, immediately after NI, and 2 h after NI, respectively. The peak height of Ristocetin-induced ROS generation was also lower in the patient group at baseline. Thus, this indicates that

blood cells in patients with renal failure have a lower ability to produce ROS upon activation with collagen or Ristocetin. Both these platelet activators act through mechanisms that, at least partly, are mediated by von Willebrand factor (vWf). In addition to two different collagen-receptors, platelets express the glycoprotein Ib complex, which interacts with vWf attached to collagen in the vascular subendothelium.[42] Ristocetin elicits vWf-dependent platelet activation.[43] An explanation for the lower response in patients with renal failure might be a dysfunction of vWf,[44] and/or abnormal Ca^{2+} mobilization in these platelets.[45] Furthermore, vWf appears to be a key protein in the exposure of P-selectin on platelets,[46] and P-selectin is, as mentioned above, the probable platelet trigger of ROS generation by granulocytes and monocytes.[41] There are reports of decreased ROS production upon specific granulocyte activation in renal failure,[47,48] and leukocyte defects might also be involved in the decreased responses observed in the current study.

Another factor that may have an impact on low ROS generation in patients with renal failure is uric acid. Increased plasma levels of uric acid are associated with renal failure and due to its antioxidative properties, uric acid has been shown to decrease luminol-enhanced chemiluminescence *in vitro*.[35] However, if uric acid in the renal patients decreased the collagen-induced luminol-enhanced chemiluminescence in the present study, the ROS production induced by the other activators would be affected as well.

In the current investigation, NI did not significantly alter the peak time of ROS generation induced by the four different compounds. NI did neither alter the amount of ROS, measured as peak height or 10 min chemiluminescence, upon activation of blood cells with fMLP, ADP or collagen. However, Ristocetin-induced ROS generation was significantly lower directly after NI in patients with renal failure, measured as peak height and 10 min chemiluminescence.

In conclusion, the present study shows that nicotine does not acutely alter the levels of the cytokines in question in healthy or renal impaired patients. However, since cytokine secretion or production was not specifically induced, these findings cannot exclude effects by nicotine on agonist-induced cytokine production. Ristocetin- and collagen-induced ROS generation tend to be lower in renal failure, and nicotine appears to acutely cause suppression on some platelet-induced activation of leukocyte ROS generation. This suppression may be amplified by defects in vWf- and/or Ca^{2+}-signaling in platelets from patients with renal failure. Thus, renal failure probably increases the susceptibility to some acute immunomodulatory effects of nicotine.

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